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**Patentanmeldung Nr. | Patent application No. | Demande de brevet n°**

96116373.0

PRIORITY DOCUMENT

Der Präsident des Europäischen Patentamts:
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For the President of the European Patent Office

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# Blatt 2 der Bescheinigung
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# Page 2 de l'attestation

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## Method of analysis of samples by determination of the distribution of specific brightnesses of particles

The present invention relates to a method for analyzing samples by measuring numbers of photon counts per defined time interval in a repetitive mode from light emitted, scattered and/or reflected by particles in the sample and determining the distribution of the number of photon counts.

The first successful studies on fluorescence intensity fluctuations were performed by Magde, Elson and Webb (Biopolymers, Vol. 13, 29-61, 1974) who demonstrated the possibility to detect number fluctuations of fluorescent molecules and established a research field called fluorescence correlation spectroscopy (FCS). FCS was primarily developed as a method for determining chemical kinetic constants and diffusion coefficients. The experiment consists essentially in measuring the variation with time of the number of molecules of specific reactants in a defined open volume of solution. The concentration of a reactant is measured by its fluorescence from a small measurement volume. The measurement volume is defined by a focussed laser beam, which excites the fluorescence, and a pinhole in the image plane of the microscope collecting fluorescence. Intensity of fluorescence emission fluctuates in proportion with the changes in the number of fluorescent molecules as they diffuse into and out of the measurement volume and as they are created or eliminated by the chemical reactions. Technically, the direct outcome of an FCS experiment is the calculated autocorrelation function of the measured fluorescence intensity.

An important application of FCS is determination of concentrations of fluorescent species having different diffusion rates, in their mixture. In order to separate the two terms in the

autocorrelation function of fluorescence intensity corresponding to translation diffusion of two kinds of particles, at least about a two-fold difference in diffusion time is needed, which corresponds to an eight-fold mass difference of particles. Furthermore, even if one succeeds in separating the two terms in the autocorrelation function of fluorescence intensity, it is yet not sufficient for determining the corresponding concentrations except if one knows the relative brightness of the two different types of particles.

Whereas conventional FCS yields rather limited information about aggregate sizes from a simple autocorrelation function of fluorescence intensity fluctuations, possible biophysical applications demand the ability to analyze complex mixtures of different species. For that purpose, Palmer and Thompson studied higher order correlation functions of fluorescence intensity fluctuations and have outlined methods for determining the number densities and relative molecular brightness of fluorescence of different fluorescent species (Biophys. J. , Vol. 52, 257-270, August 1987). Their technique may in principle proof useful in detecting and characterizing aggregates of fluorescent-labeled biological molecules such as cell surface receptors, but has a major disadvantage of being rather complex, so that data processing of an experiment including the calculation of high-order correlation functions lasts hours.

A considerably less complicated method than calculation of high order auto-correlation functions for characterizing mixtures of fluorescent species of different specific brightness is calculation of higher order moments of fluorescence intensity out of the experimentally determined distribution of the number of photon counts. This method was presented by Qian and Elson (Biophys. J., Vol. 57, 375-380, February 1990; Proc. Natl. Acad. Sci. USA, Vol. 87, 5479-5483, July 1990). In their demonstration experiments signal acquisition times of about 7 minutes were used for a relatively favourable experimental system of two kinds of fluorescent particles which differed by 30-fold in

their specific brightness, the mixture of monomers and 30-mers. The method of moments is relatively simple and fast in calculations, but it allows to determine only a limited number of unknown parameters characterizing the sample because usually only about three or four first moments of fluorescence intensity can be calculated from the experiment with precision sufficient for further analysis. Because of this reason, the method of moments is hardly suitable for characterizing complex samples or selecting between competing models of the sample or checking whether the given model is appropriate.

One object of the invention is to obtain reliable information about a sample containing particles emitting, scattering and/or reflecting photons, which renders possible an analysis of the sample with respect to certain ingredients or with respect to certain states of the sample.

Another object of the present invention is to substantially extend the useful information obtainable from the experimentally determined distribution of the number of photon counts.

The objects of the present invention are solved with the method having the features of claim 1.

What is meant by the term "specific brightness" of particles in the sense of the present invention is a physical characteristic expressing in what extent a given type of particles is able to emit, scatter and/or reflect light. It is thought to characterize single particles and therefore the value of specific brightness is not depending on concentration of the particles, neither on the presence of other particles. Thus, a change of the total count rate of photons emitted, scattered and/or reflected from the measurement volume, if only due to a change in concentration of the total number of particles, does not influence the measured value of specific brightness and the value of the ratio of numbers of particles of different types determined by the present invention. Specific brightness of a

type of particles is usually expressed in terms of the mean count rate per particle which is a weighted average of the count rate over coordinates of the particle in the measurement volume.

The importance of the present invention for the analysis of samples may be illustrated by the following, non-limiting example: Assuming that a solution contains a quantity (a) of one type of particles (A) with a respective specific brightness (Ia) and a quantity (b) of another type of particles (B) with a respective specific brightness (Ib), the overall count rate of photons emitted by the solution depends on the expression Ia*a + Ib*b. Thus, by mere determination of the overall count rate, it is not possible to dissolve the value of a and/or b. Generally, in fluorimetric measurements, the overall count rate of at least one type of particles is determined in an independent experiment. If the total number a+b of particles does not change with respect to this measurement, the ratio a/b or its inverse can be determined by mere determination of the overall count rate of the mixture in a second measurement. However, the assumption, that the total number a+b does not change between the two measurements, is often wrong. For example, adsorption effects of particles to surfaces may occur. Fluorimetric measurements cannot verify the total number of particles a+b independently. The present invention overcomes these restrictions. From one measurement, the numbers of particles a and b can be determined without any prior information of their respective specific brightnesses.

It is to be understood that the following description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of skill in the art upon reviewing the following description. By way of example, the invention will be described primarily with reference to measuring numbers of photon counts from light emitted by fluorescently labelled particles in a sample. For example, in some embodiments it may be desirable to measure numbers of photon counts of other origin than fluorescence.

The present invention provides a method for calculating the expected distribution of the number of photon counts corresponding to given real equipment and samples of given composition. The ability to predict the distribution of the number of counts corresponding to samples of given composition allows, when studying samples of unknown composition, to find out the model of the sample yielding the closest fit between the calculated and the experimentally determined distribution of the number of photon counts. What is meant by the composition of the sample here is the specific brightness and concentration of fluorescent particles present in the sample. For example, a solution of a single fluorescent dye is characterized by two parameters: the concentration and specific brightness of the dye molecules. A mixture of two fluorescent dyes is characterized by four parameters: the concentrations and specific brightnesses of the two kind of molecules. A complex mixture can be characterized by the distribution function of concentration versus specific brightness of particles. Conveniently, concentration is expressed as the mean number of particles per measurement volume, and specific brightness of particles is expressed as the mean count rate per particle.

From the other side, the distribution of the number of photon counts depends not only on composition of the sample, but also on equipment: first of all, on the spatial brightness function characteristic to the optical set-up, and on some characteristics of the detector like its dark count rate, its dead time and probability of afterpulsing. In the interest of a high quality of analysis, which is indicated by achieving a close fit between the experimentally determined and the calculated curves, it is preferred to characterize the equipment adequately.

Claim 1 does not cover the method described by Qian and Elson, as their method compares estimated and calculated moments of the distribution of light intensity, but not directly the distribution of the number of photon counts. Qian and Elson's teaching misses the teaching of the present invention.

According to the invention, a new quality of analysis of samples containing particles which emit, scatter and/or reflect light becomes possible. In a first step, numbers of photon counts from light emitted by the particles in the sample are measured per defined time interval in a repetitive mode. A series of different time intervals can also be used for a more complex analysis. In a second step of the method according to the invention, the distribution of the number of photon counts is determined, which means that it is determined how many times one has obtained a certain number of photon counts. In a third step, the experimentally determined distribution of photon counts is analyzed directly without intermediate steps to obtain the distribution of specific brightnesses of the particles in the sample.

In the sense of the present invention, particles are preferably luminescently labelled or unlabelled molecules or macromolecules, or dye molecules, molecular aggregates, complexes, vesicles, cells, viruses, bacteria, beads, or mixtures thereof.

In a further preferred embodiment, the particles each carry a number of binding sites for luminescent particles. Luminescent particles can directly or via secondary molecules bind to these binding sites. Since highly luminescent particles are generated when many luminescent particles bind to the binding sites of the first particles, the method according to the invention is able to distinguish easily between particles with a large difference in luminescence intensity, so that even a small amount of bound luminescent particles can be measured in presence of an excess concentration of free luminescent particles. This embodiment provides a new analysis of particles which do not carry a luminescent label by binding to a second particle which is luminescently labelled, but whose brightness does not change upon binding. A commercially very important application of this method is the measurement of fluorescently labelled antibodies binding to an antigen, while the antigen is binding to at least one of the multiple binding sites of the

particle which is preferably a bead, or vice versa. The method can also be applied to other types of interactions such as nucleic acid hybridization or protein/nucleic acid interaction. The invention can also be applied for the analysis of distribution characteristics of said particles, such as for quality control and process control of polymers or oligomeric suspensions of particles. In addition, surface areas of particles can be analyzed as well as distributions of surface areas of particles.

In a preferred embodiment, one type of particle, subsequently denoted A, carries more than one binding site. Another, luminescent type of particles, subsequently denoted C, can bind (i) either directly to at least one of the binding sites of particle A, or (ii) binds to at least one binding site of a molecule B, which in turn bind to at least one of the binding sites of particle A. These bindings may be mediated either by naturally occurring binding sites on the particles, or mediated by introduction of specific binding sites to the particles A, B and/or C. Since in both cases more than one of the particles of type C may bind to particle A, the complex will emit more photons than free particles of type C. This embodiment provides a convenient way to measure binding of particles of type B to a particle of type C or A, although the particle of type B is not luminescently labelled.

In a further preferred embodiment, the measurement volume is only a part of the total volume of the sample and said particles are diffusing and/or being actively transported into and out of said measurement volume and/or the sample is actively transported and/or optically scanned. If the fluorescent particles are sufficiently small, then diffusion is fast enough for data acquisition from a great number of independent measurement volumes, and data acquisition using time averaging is nearly identical to ensemble averaging. However, if the characteristic time of diffusion is substantially longer than the time interval necessary for measuring fluorescence intensity (which is usually

10 to 50 μs), then active transport (flow or scanning) can considerably save time of data acquisition.

A sample is usually characterized by values of concentration and specific brightness of one or more types of fluorescent particles. In cases when one or more of these values are known beforehand, the goal of analysis is to determine unknown values, either those of concentration, or specific brightness, or both.

Two alternative methods for selecting the model yielding a fit between the experimentally determined and calculated distributions of the number of photon counts can be used. In one embodiment, the well-known least squares fitting method, where the sample is described by a finite (usually small) number of parameters, can be employed. The purpose is to find values of the parameters yielding the closest fit between the experimental and the calculated curves. According to the invention, values of concentrations and/or specific brightnesses of a number of types of fluorescent particles can be estimated. In a further embodiment, another general method called inverse transformation with linear regularization (ITR) can be employed. ITR describes the sample using a semi-continuous distribution function of particles versus their specific brightness, and searches for the closest fit demanding that the solution is a smooth function. (For the method of ITR, see, e.g., W. H. Press, S. A. Teukolsky, W. T. Vetterling, B. P. Flannery, Numerical recipes in C: the art of scientific computing, second edition, Cambridge University Press, 1992, p. 808)

In the following, the invention is further illustrated in a non-limiting manner. Particularly, it is described how the expected distribution of the number of photon counts is calculated.

A first step is the calculation of the probability distribution of the number of photon counts from a spatial section of the measurement volume with a constant value of spatial brightness. This can be done by using a single type of fluorescent parti-

cles. It is well known that the probability distribution of the number of particles in an open volume is Poissonian. Also, if the number of particles inside the spatial section is given, the number of detected photons per sampling time interval is Poisson distributed. Consequently, the overall distribution of the number of photon counts corresponding to the spatial section of constant brightness and an ideal detector is compound Poissonian.

As the next step, one may study the case in which the measurement volume is divided into a number of spatial sections of constant brightness. If the values of volumes and spatial brightnesses in each of the sections are known, the distribution of the number of photon counts corresponding to each section can be calculated separately. All these distributions are compound Poissonian. Furthermore, if distributions of the number of photon counts for all sections were known, the overall distribution can be calculated using the fact that the total number of counts is the sum of the number of counts originating from different sections of the measurement volume.

As the following step, one may study the mixture of types of fluorescent particles having different values of specific brightness. Each spatial section of the measurement volume can be divided into a number of abstract subsections each containing only particles of a single type. A similar procedure can be applied now as described above for spatial sections of the measurement volume in order to calculate the overall distribution of the number of photon counts.

An experimentally determined distribution of the number of photon counts is ruled not only by properties of the light beam, but is influenced also by nonideal properties of the photon detector. Statistically, the dark counts of the detector behave in the same manner as photon counts from background light of constant intensity. Their contribution are photon counts of Poisson distribution. Also, the way how the dead time of the

detector and its afterpulsing distort the distribution of photon counts are known from literature on photon statistics (see e.g. B. Saleh, Photoelectron Statistics, Springer, Berlin, 1978).

In summary, the expected distribution of the number of photon counts is determined, from one side, by characteristics of the sample (concentrations and specific brightnesses of fluorescent particles of different kind), and, from the other side, by characteristics of the equipment (the sampling time interval, the spatial brightness function, the background count rate, the dead time and the afterpulsing probability of the detector).

In one embodiment, both the dead time and the afterpulsing probability of the detector are determined from experiments in which the distribution of the number of photon counts corresponding to light of constant intensity is determined. The experimental parameters of the detector may also be determined by measuring numbers of photon counts per defined time intervals in a repetitive mode while the detector is exposed to high frequency laser pulses. Correction for the dead time of the detector may be performed on the basis of a formula derived by Cantor and Teich (J. Opt. Soc. Am. 65, 786,1975; see also B. Saleh, p. 272-277). Mathematics of afterpulsing is simple: each photon pulse can be followed by another (artificial) pulse; this happens usually with a constant probability.

According to the invention, it is preferred that the spatial brightness function is characterized using experiments on a single type of particles. For example, if the laser wavelength 514.5 nm is used, then a solution of Rhodamine 6G is a convenient sample which can be used for characterizing the spatial brightness function.

What characteristics of the spatial brightness function can be employed when calculating the expected distribution of the number of counts are values of volumes of the sections of the measurement volume corresponding to a selected set of values

of the spatial brightness. Typically, a set of twenty values of the spatial brightness positioned at a constant distance from each other in the logarithmic scale have been selected, covering two orders of magnitude. Contribution from the lower brightness areas can be accounted for by a single parameter, their relative contribution to fluorescence intensity. Intensity fluctuations of this light can be neglected. Because of the large number of the sections of the measurement volume, it would be less preferred to consider volumes corresponding to each of the sections as independent variables. It is convenient to consider them as variables depending on a few other parameters, and determine the values of these parameters which yield the closest fit between the experimentally determined and the calculated distribution of the number of photon counts. Conveniently, a relatively simple model of the optical set-up is applied, which is not accounting for aberrations of the optics used, and which determines volumes of the sections of the measurement volume. For instance, the volumes of the sections depend on values of the convergence angle of the laser beam and the size of the pinhole.

In fluorescence studies, it may be advantageous to take measures for reducing the background count rate, arising from Raman scattering in the solute material and dark count rate of the detector, with respect to the count rate per particle. In particular, it is in some cases preferred to use measurement volumes smaller than 100 $\mu m^3$, more preferably about 1 $\mu m^3$. Advantageously, the high signal to background count rate and the small optical measurement volume may be achieved by using at least one high-aperture microscope objective in a confocal manner for both focussing the incident laser beam and collecting light emitted, scattered and/or reflected by particles in said sample.

In a preferred embodiment of the method, multiple photon excitation is used to excite a particle. Multiple photon excitation means that the sum, difference or any combination of wave frequences of two, three or more photons is used for excitation

of e.g. luminescence or second order Raman scattering. Such an excitation scheme has an advantage in the sense that the excitation probability is not linearly dependent on excitation intensity, but on the second or higher power. Thus, the multiple photon excitation is mostly limited to the volume of the laser focus, whereas outside the laser focus no spurious excitation is generated. The present invention profits from such an excitation scheme in the sense that less background is generated compared to single photon excitation, and that there is no pinhole necessary to restrict the measurement volume. Thus, the pinhole diameter and its imaging on the detector do not enter as modelling parameters in the spatial brightness function any more.

In a further preferred embodiment, near field optical microscopy is used for focussing the excitation light of the particles, and/or collecting the light emitted by the particles. Near field optical microscopy means here that the light passes through an aperture with at least one of its dimensions being smaller than the wavelength of the light used and which is in direct contact to the measurement volume. The aperture may consist of an opaque layer with at least one hole of said diameter or at least one slit of appropriate width and/or a tapered glass fiber or wave guide with a tip diameter of said width, optionally coated with an opaque layer outside.

Another preferred embodiment combines near field optical microscopy for the excitation light path, and conventional optical microscopy for the emission light path, or vice versa. The present invention profits from such a realization in the sense that the size of the measurement volume is reduced compared to conventional confocal microscopy. Thus, the present invention can be used to measure higher particle concentrations as with other optical schemes.

According to the invention, it may in some cases be preferred to select the width of the sampling time interval in such a way

that in average more than one, preferably one to ten, photon counts per particle are yielded.

It may further be preferred that the width of the time interval is in average smaller than the characteristic correlation time of light intensity fluctuations.

If more than two unknown parameters of the sample have to be estimated, it is preferred to have no more than a few, preferably about one particle per measurement volume.

In one embodiment, at least one individual particle is statistically analyzed in terms of its specific brightness which may fluctuate or otherwise change.

The method of the present invention is particularly advantageous since not only that information losses and distortions are kept minimal. One new quality attainable by the present invention is that the data processing depends less from a definite mathematical model of the sample than do the other techniques which are state of the art. This means that the method is more reliable in terms of long term stability of an instrumental realization, and that any disturbation of the measurement volume by e.g. turbid samples or particles inside the laser beam does not significantly influence experimental results.

A further new quality attainable by the present invention is that the signal-to-noise ratio is much better compared to the techniques of the prior art. This means that experiments can be made within significantly shorter time (up to 100 fold shorter) than previously, showing the same signal-to-noise ratio as previous long term experiments. Since photo-bleaching of fluorescent molecules or fluorescently labelled molecules is an unsolved problem so far with any applied measurement technique in this field, especially when applied to cells, one is restricted to short measurement times. Thus, compared to the

prior art, the present invention is advantageous for measurements inside cellular systems.

In one preferred embodiment, the present invention is realized in the field of fluorescence intensity fluctuation studies. The optical equipment is a conventional confocal FCS microscope equipped with a cw laser of visible light. The excitation laser beam is focussed into a sample which is a homogeneous water solution of a low concentration, typically in the nanomolar range, of fluorescent material. Fluorescence emission from a microscopic confocal volume of about 1 $\mu m^3$ is collected on a photon detector. The measurement time which is typically 1 to 60 seconds is divided into hundreds of thousands time intervals of typical width of 10 to 50 $\mu s$. The highest number of photon counts typically obtained in this experimental realization of the invention herein described is between 10 and 100.

The nature and advantages of the invention may be better understood on the basis of the following example where a mixture of rhodamine dyes is analyzed. Figures 1 to 7 illustrate consecutive steps of the analysis and their results.

Fig. 1. Distributions of the number of photon counts experimentally determined at constant light intensities, time interval of 10 $\mu s$ and data collection time of 50 s. From curve fitting, the dead time of the detector was estimated to be 28 ± 4 ns; afterpulsing probability 0.0032 ± 0.0008. In the lower graph, weighted residuals of the curve fitting are presented.

Fig. 2. Distribution of the number of photon counts experimentally determined for a solution of rhodamine 6G at time interval of 40 $\mu s$ and data collection time of 50 s.

Fig. 3. Normalized sizes of volumes of the twenty spatial sections of the measurement volume of the highest brightness. Section 0 corresponds to the maximal value of the spatial brightness while section 19 corresponds to about 100 times lower

brightness. Sizes of volumes are determined from experiments on single fluorescent species.

Fig. 4. Residuals of curve fitting corresponding to an experiment on rhodamine 6G solution (the experiment graphed by Fig. 2).

Fig. 5. Distribution of the number of photon counts experimentally determined for three samples at time interval of 40 $\mu$s and data collection time of 50 s. The distribution corresponding to rhodamine 6G is the same as in Fig. 2.

Fig. 6. The results of the inverse transformation with linear regularization applied to the data of Fig. 5.

Fig. 7. Residuals corresponding to analysis of an experiment on the mixture solution of rhodamine 6G and tetramethylrhodamine (measured data in Fig. 5). Graph a: expected curve was obtained by inverse transformation with linear regularization. Graph b: expected curve was obtained by the least squares fitting of the experimental data. Graph c: residuals of the least squares curve fitting under a wrong assumption of single species.

As the first preparatory step of analysis, the dead time and the afterpulsing probability of the photon detector are estimated. This was done by determining the distribution of the number of photon counts under illumination of the detector by light of constant intensity. Since the dead time distortions are most noticeable at high count rates while the afterpulsing distortions are better resolved at low count rates, the values of the dead time and the probability of afterpulsing were determined by jointly fitting distributions of the number of photon counts determined at a relatively high and at a relatively low illumination intensity. The experimentally determined count number distributions are presented in Fig. 1, together with residuals of the curve fitting. Values of the estimated

parameters for the photon detector which have been used are: dead time 28 ns, afterpulsing probability 0.003.

The background count rate of the equipment is determined by measuring the count rate when the sample holder is filled with pure water.

As the second preparatory step, the spatial brightness distribution corresponding to a given optical set-up was characterized. For that purpose, the distribution of the number of photon counts corresponding to a solution of rhodamine 6G was experimentally determined (Fig. 2). If the spatial brightness distribution is appropriately characterized, then the calculated curve fits the experimental curve. In order to numerically calculate the expected distribution of the number of photon counts, values of twenty one parameters characterizing the spatial profile are needed in our program: twenty sizes of volumes corresponding to twenty spatial sections of the highest values of spatial brightness, and the relative contribution to fluorescence light originating from areas of lower spatial brightness. In order to calculate values of these unknown parameters, a simple model of the optical equipment not accounting for aberrations was taken into use. As illustrated by Fig. 3, the determined sizes of the twenty volumes are reproducible, even if other species than rhodamine 6G are used.

Having determined values of the twenty one parameters characterizing the spatial brightness distribution in the way just described above, the calculated distribution of the number of photon counts indeed fits the experimentally curve, see Fig. 4.

After the preparatory steps described above the equipment is ready for analysis. In Fig. 5, distributions of the number of photon counts corresponding to three different samples are presented. In Fig. 6, the results of the inverse transformation with linear regularization are graphed. Both spectra corresponding to single species (rhodamine 6G or tetramethylrhodamine)

have a single peak, but the peaks are centered at different values of specific brightness. The peak of rhodamine 6G is situated at about 108 kHz/molecule, whereas the peak of tetramethylrhodamine is centered at about 37 kHz/molecule. This indicates that a rhodamine 6G molecule is about 3 times brighter than a tetramethylrhodamine molecule. The spectrum corresponding to the mixture of the two species has two peaks centered indeed near the values obtained for the two species separately.

Fig. 7 illustrates the residuals corresponding to the measurements of the mixture of rhodamine 6G and tetramethylrhodamine. Different methods of data processing yield slightly different fit curves (and different residuals). The upper graph corresponds to the spectrum of specific brightness obtained by inverse transformation with linear regularization. The middle graph corresponds to the fit curve obtained assuming two species. These two graphs are nearly identical. The experimentally determined distribution of the number of photon counts can formally be fitted under the wrong assumption of single species, which is shown in the lower graph, but the fit curve is obviously apart from the experimental one.

## CLAIMS

1. A method for analyzing samples by measuring numbers of photon counts per defined time interval in a repetitive mode from light emitted, scattered and/or reflected by particles in said sample, and determining the distribution of the number of photon counts per said time intervals, characterized in that the distribution of specific brightnesses of said particles is determined from said distribution of the number of photon counts.

2. A method according to claim 1, wherein said particles are luminescently labelled or unlabelled molecules or macromolecules, or dye molecules, molecular aggregates, complexes, vesicles, cells, viruses, bacteria, beads, or mixtures thereof.

3. A method according to claim 1 and/or 2, wherein said particles each carry a number of binding sites for luminescent particles.

4. A method according to at least one of the claims 1 to 3, wherein the measurement volume is only a part of the total volume of the sample and said particles are diffusing and/or being actively transported into and out of said measurement volume and/or the sample is actively transported and/or optically scanned.

5. A method according to at least one of the claims 1 to 4, wherein the parameters of the spatial brightness function characteristic for the optical set-up are determined by

measuring numbers of photon counts per defined time intervals in a repetitive mode from light emitted, scattered and/or reflected by a single type of particles.

6. A method according to at least one of the claims 1 to 5, wherein at least one high-aperture microscope objective is used in a confocal manner for both focussing the incident laser beam and collecting light emitted, scattered and/or reflected by particles in said sample.

7. A method according to at least one of the claims 1 to 6, wherein the size of the pinhole positioned in the focal plane of the microscope is used as a modelling parameter of the spatial brightness function.

8. A method according to at least one of the claims 1 to 7, wherein the convergence angle of the incident laser beam is used as a modelling parameter of the spatial brightness function.

9. A method according to at least one of the claims 1 to 8, wherein multiple photon excitation is used to excite said particles in said sample.

10. A method according to at least one of the claims 1 to 9, wherein the measurement volume is restricted by the use of near field optical microscopy, or combinations thereof with conventional microscopy optics.

11. A method according to at least one of the claims 1 to 10, wherein the concentration and/or specific brightness of at least one type of said particles is determined.

12. A method according to at least one of the claims 1 to 11, wherein said distribution of the number of photon counts is fitted using a priori information on the sample.

13. A method according to at least one of the claims 1 to 12, wherein said distribution of the number of photon counts is processed by applying an inverse transformation with linear regularization.

14. A method according to at least one of the claims 1 to 13, wherein the experimental parameters of the detector, in particular the dead time and the afterpulsing probability of the detector, are determined by measuring numbers of photon counts per defined time intervals in a repetitive mode while the detector is exposed to light of constant intensity or high frequency laser pulses.

15. A method according to at least one of the claims 1 to 14, wherein the background count rate of the equipment is determined.

16. A method according to at least one of the claims 1 to 15, wherein the width of said time interval is in average smaller than the characteristic correlation time of light intensity fluctuations.

17. A method according to at least one of the claims 1 to 16, wherein the width of said time interval is selected to yield in average more than one, preferably 1 to 10, photon counts per said particle.

18. A method according to at least one of the claims 1 to 17, wherein the concentration of the sample or the size of the measuring volume is selected to have in average no more than a few, preferably about one particle per measuring volume.

19. A method according to at least one of the claims 1 to 18, wherein at least one individual particle is statistically analyzed in terms of its specific brightness which may fluctuate or otherwise change.

## Abstract

A method for analyzing samples by measuring numbers of photon counts per defined time interval in a repetitive mode from light emitted, scattered and/or reflected by particles in said sample, and determining the distribution of the number of photon counts per said time intervals, characterized in that the distribution of specific brightnesses of said particles is determined from said distribution of the number of photon counts.

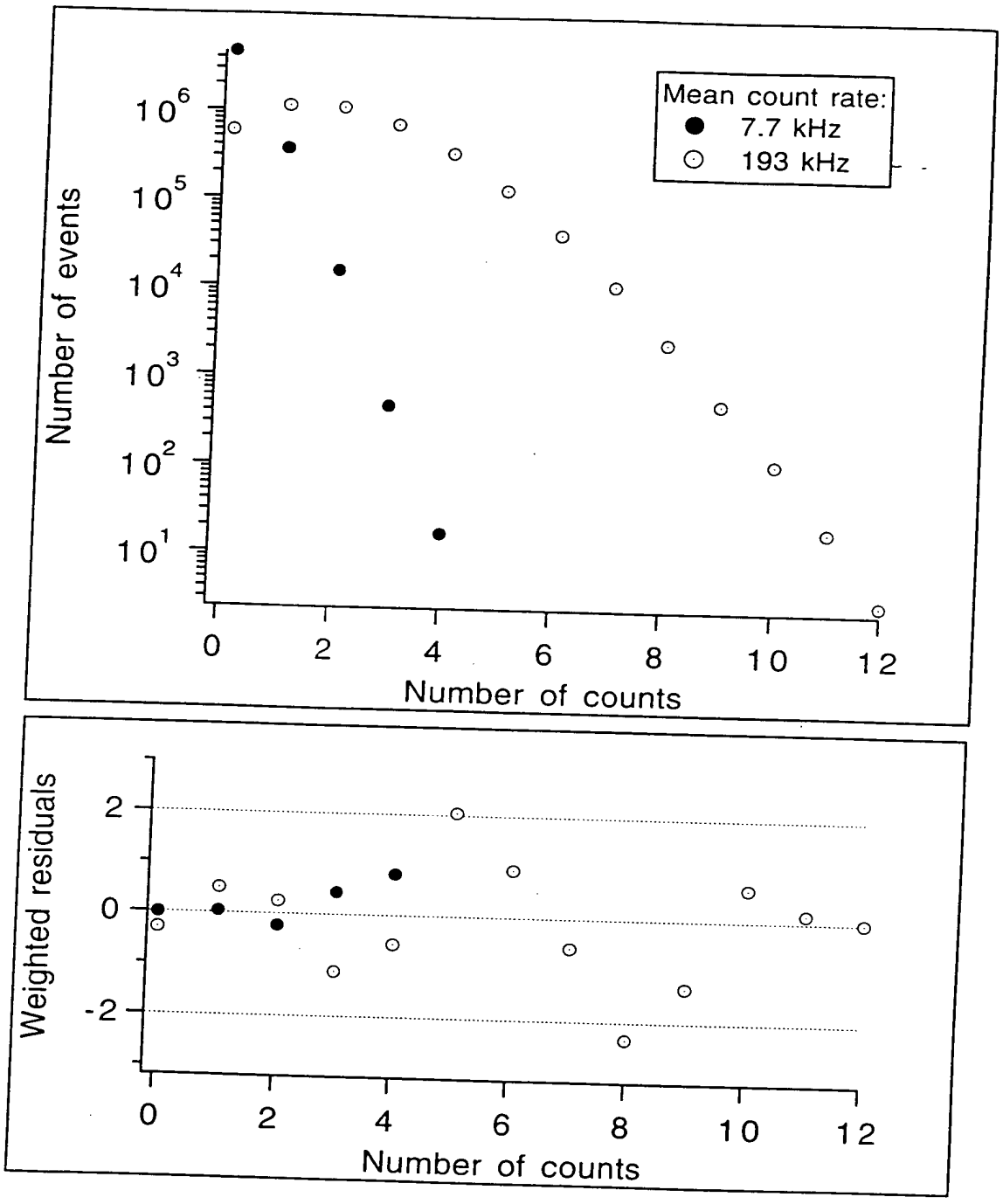
Mean count rate:
7.7 kHz
193 kHz
Number of events
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
0
2
4
6
8
10
12
Number of counts
Weighted residuals
2
0
-2
0
2
4
6
8
10
12
Number of counts

Fig. 1

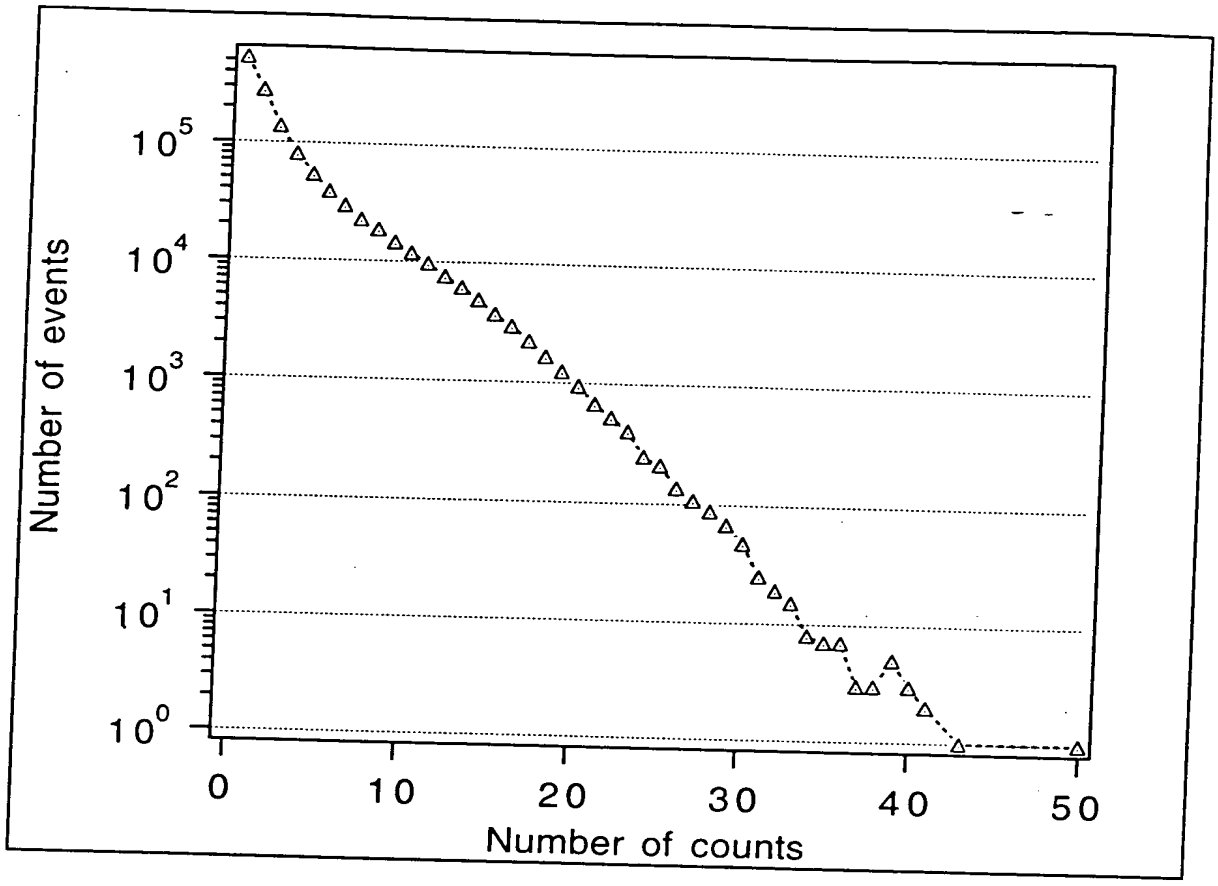
Number of events
10^5
10^4
10^3
10^2
10^1
10^0
0
10
20
30
40
50
Number of counts

Fig. 2

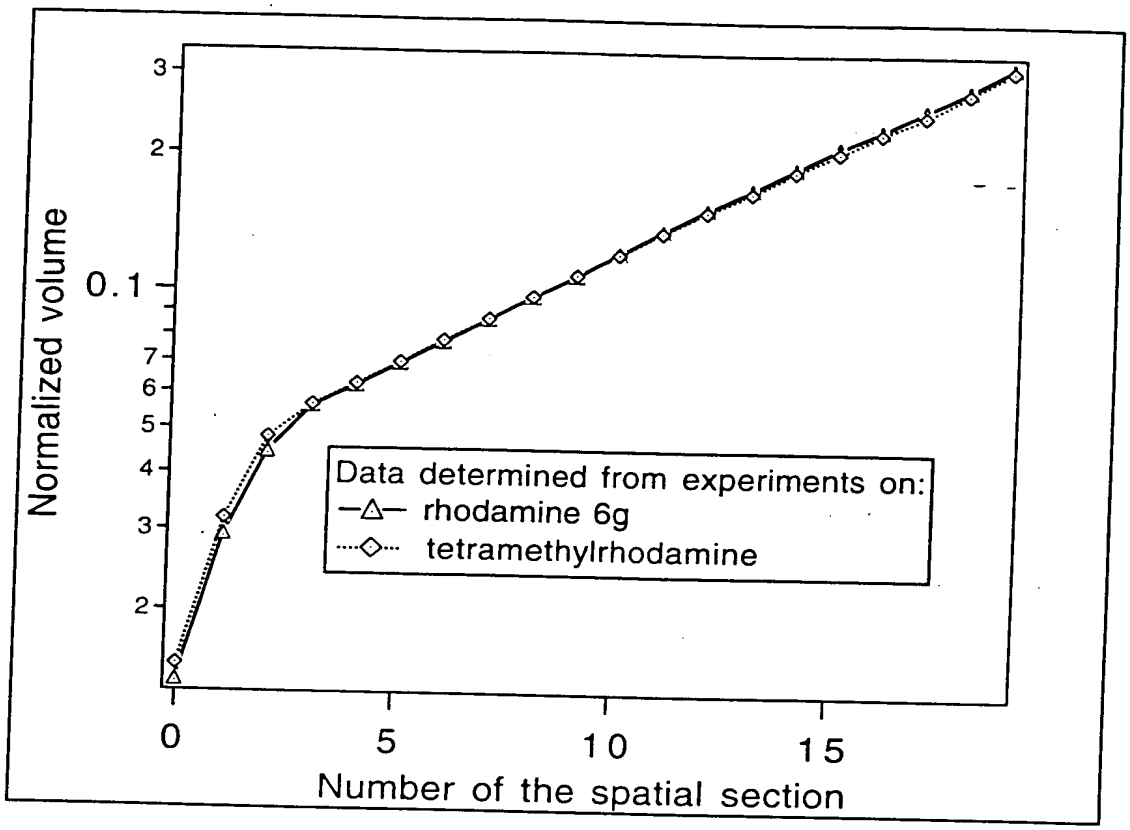
Normalized volume
3
2
0.1
7
6
5
4
3
2
0
5
10
15
Number of the spatial section
Data determined from experiments on:
rhodamine 6g
tetramethylrhodamine

Fig. 3

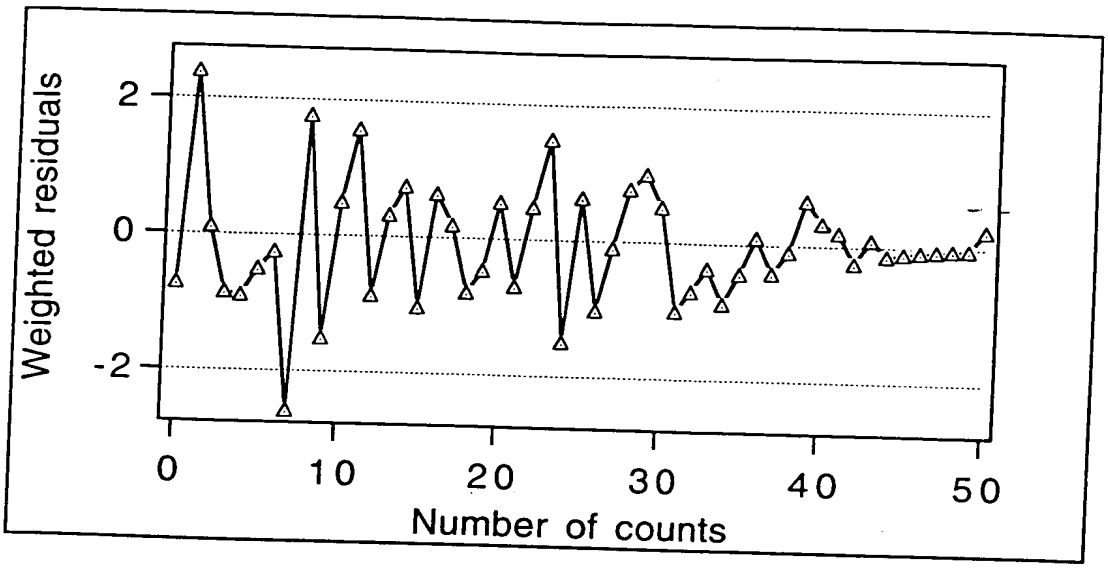


Fig. 4

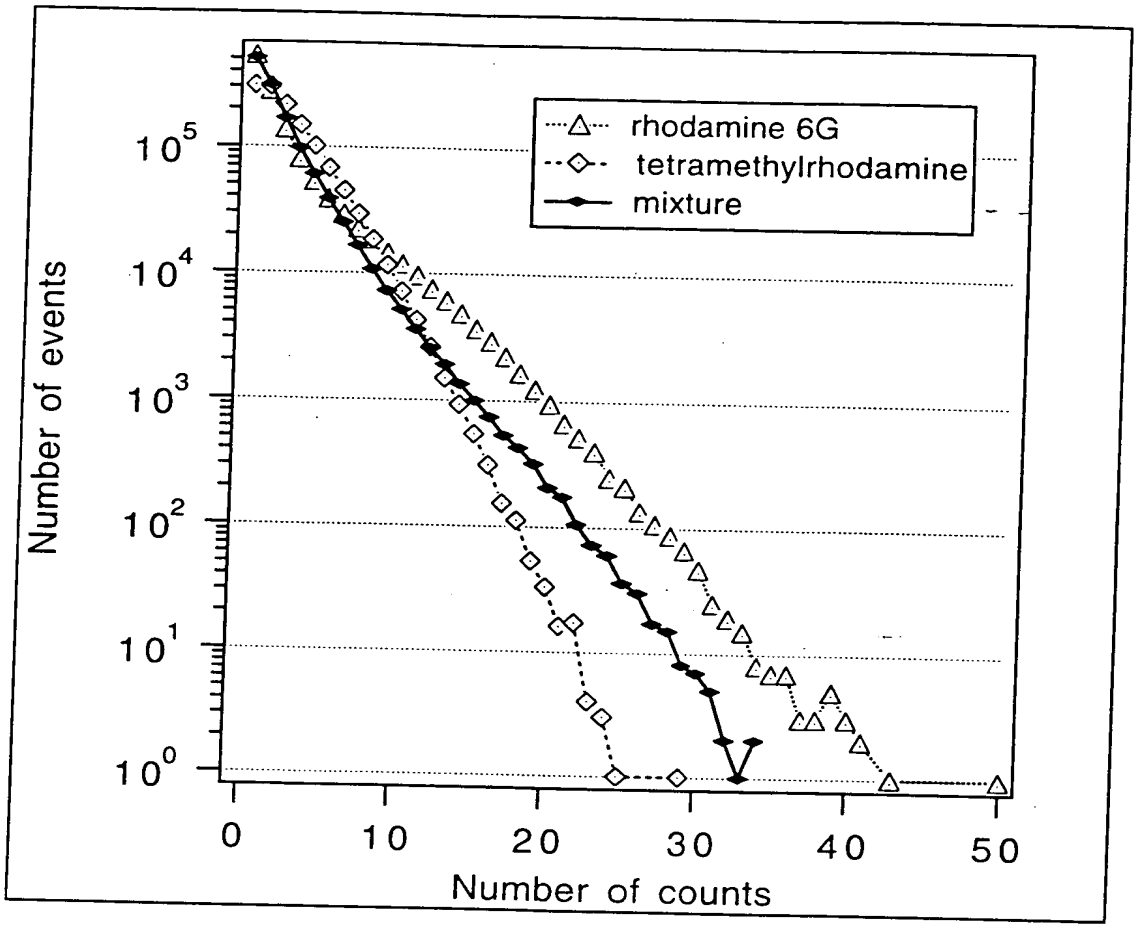
rhodamine 6G
tetramethylrhodamine
mixture
Number of events
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
0
10
20
30
40
50
Number of counts

Fig. 5

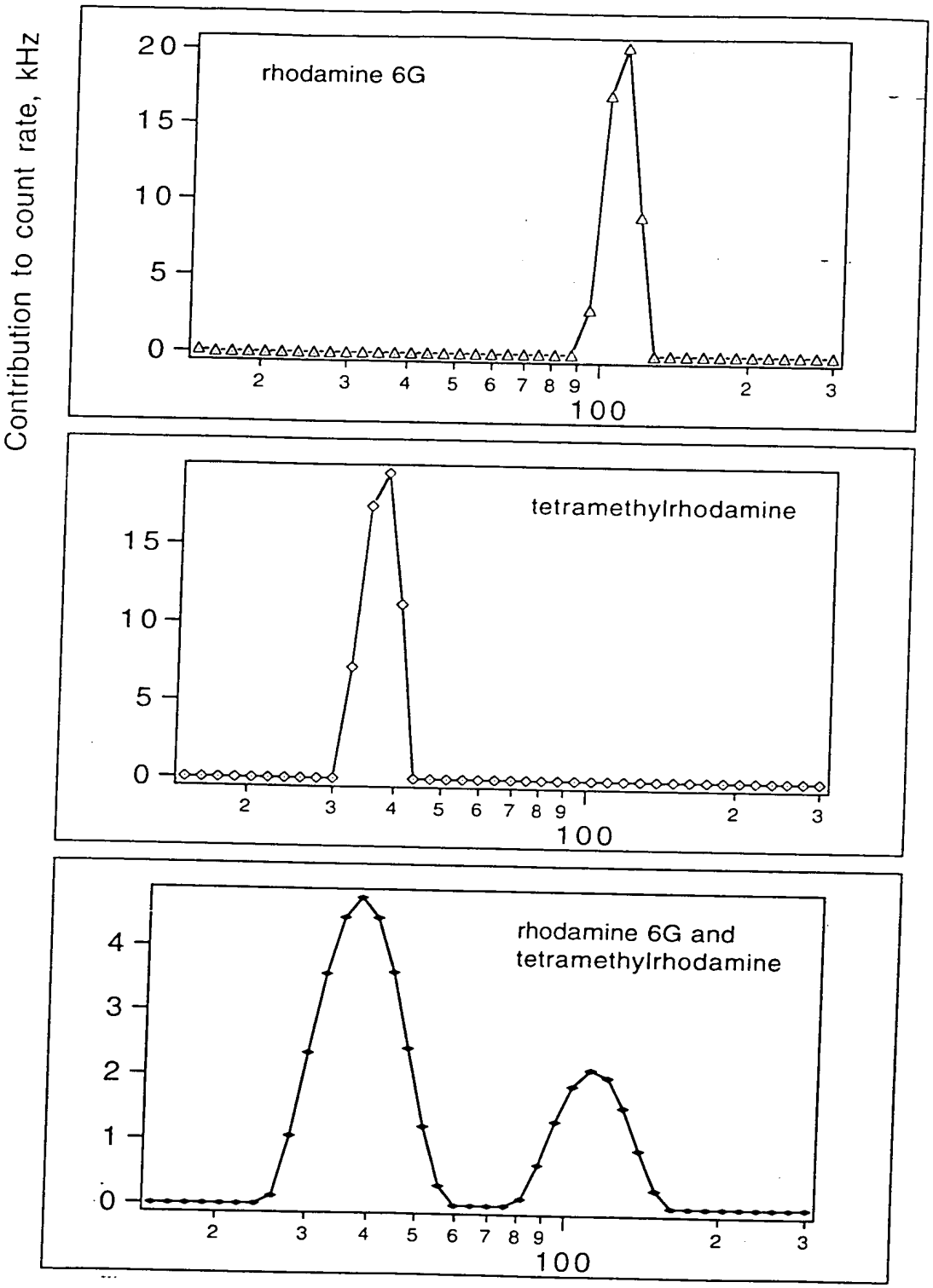
Contribution to count rate, kHz
rhodamine 6G
tetramethylrhodamine
rhodamine 6G and
tetramethylrhodamine
Molecular brightness, kHz/molecule

Fig. 6

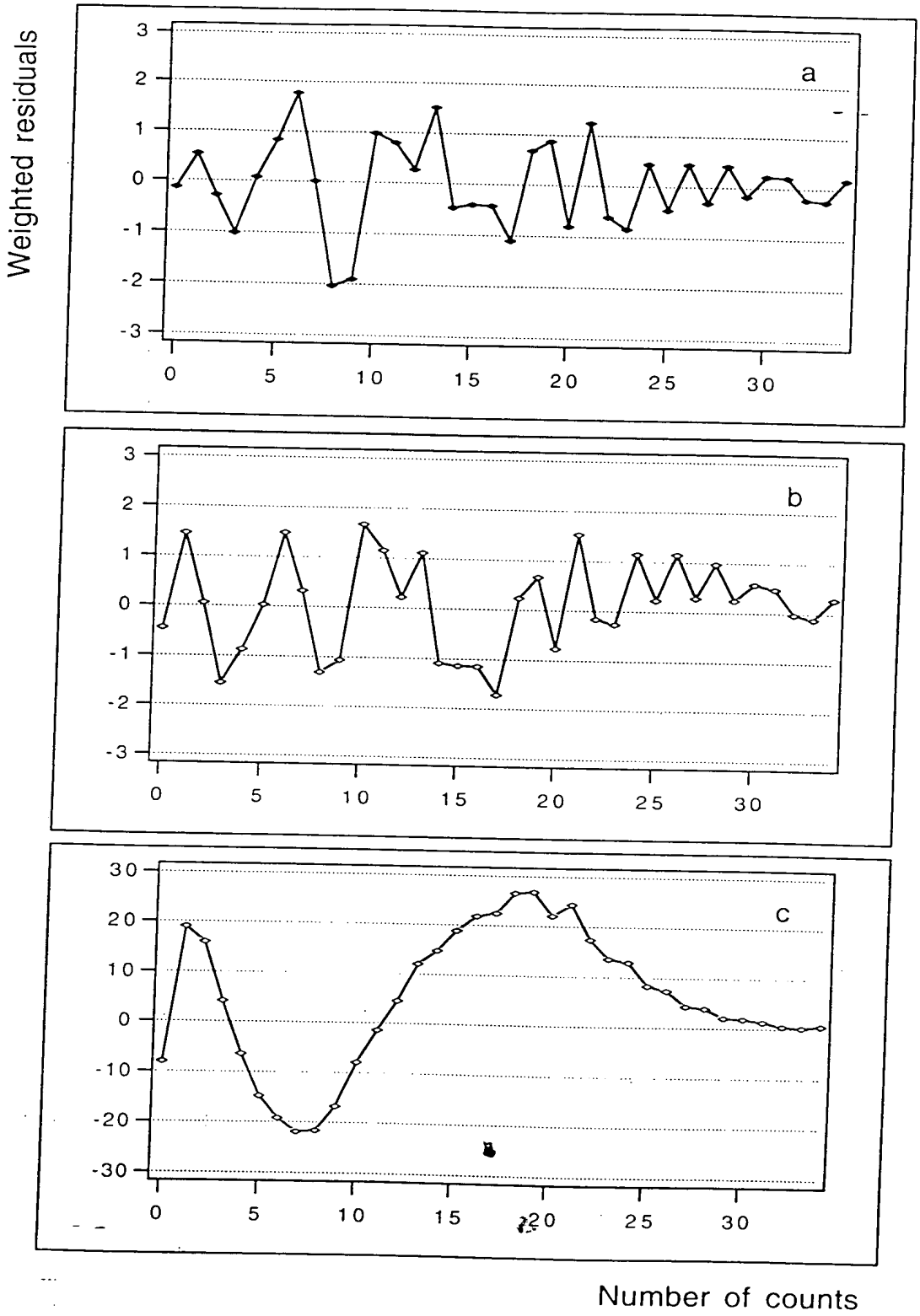


Fig. 7